ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 2003, p. 3384–3392 Vol. 47, No. 11
0066-4804/03/$08.00+0 DOI: 10.1128/AAC.47.11.3384–3392.2003

Mitochondrial and Metabolic Effects of Nucleoside Reverse Transcriptase Inhibitors (NRTIs) in Mice Receiving One of Five Single- and Three Dual-NRTI Treatments

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Received 7 November 2002/Returned for modification 17 February 2003/Accepted 15 July 2003

Although treatments with nucleoside reverse transcriptase inhibitors (NRTIs) can modify fat metabolism and fat distribution in humans, the mechanisms of these modifications and the roles of diverse NRTIs are unknown. We studied the mitochondrial and metabolic effects of stavudine (d4T), zidovudine (AZT), didanosine (ddI), lamivudine (3TC), zalcitabine (ddC), and three combinations (AZT-3TC, d4T-3TC, and d4T-ddI) in mice treated for 2 weeks with daily doses equivalent to the human dose per body area. Concentrations of AZT and d4T in plasma were lower when these drugs were administered with 3TC or ddI. Whatever the treatment, mitochondrial DNA was not significantly decreased in muscle, heart, brain, or white adipose tissue but was moderately decreased in liver tissue after the administration of AZT, 3TC, or d4T alone. Blood lactate was unchanged, even when NRTIs were administered at supratherapeutic doses. In contrast, the level of plasma ketone bodies increased with the administration of AZT or high doses of d4T but not with ddC, 3TC, or ddI, suggesting that the thymine moiety could be involved. Indeed, the levels of plasma ketone bodies increased in mice treated with β-aminoisobutyric acid, a thymine catabolite. Treatment with AZT, d4T, or β-aminoisobutyric acid increased hepatic carnitine palmitoyltransferase I (CPT-I) mRNA expression and the mitochondrial generation of ketone bodies from palmitate. In conclusion, therapeutic doses of NRTIs have no or moderate effects on mitochondrial DNA and no effects on plasma lactate in mice. However, AZT and high doses of d4T increase the levels of hepatic CPT-I, mitochondrial fatty acid β-oxidation, and ketone bodies, and these catabolic effects are reproduced by β-aminoisobutyric acid, a thymine metabolite.

Nucleoside reverse transcriptase inhibitors (NRTIs), including zidovudine (AZT), stavudine (d4T), didanosine (ddI), zalcitabine (ddC) and lamivudine (3TC), are used in the treatment of human immunodeficiency virus (HIV) infections. In combination with HIV protease inhibitors, NRTIs afford outstanding benefits in terms of HIV-induced morbidity and mortality (11). However, NRTIs can induce side effects of differing severity and frequency (6, 12, 26). Liver failure, lactic acidosis, or pancreatitis may be severe and sometimes fatal, but these occur in only a few patients (7, 13, 17, 48). Lipodystrophy and hyperlactatemia are less severe but much more frequent (14, 35).

Mitochondrial dysfunction is a key event leading to some NRTI-induced adverse effects, including liver failure, lactic acidosis, myopathy, and neuropathy (5, 37, 38). Mitochondrial dysfunction is mainly ascribed to NRTI-mediated impairment of mitochondrial DNA (mtDNA) replication. However, other mitochondrial targets may also exist, since NRTI-induced mitochondrial dysfunction and metabolic disturbances can occur without significant mtDNA depletion (2, 4, 33, 43). Furthermore, not all effects of NRTIs are due to mitochondrial dysfunction. Instead, we recently observed that high doses of d4T paradoxically increased hepatic mitochondrial fatty acid oxidation in fasted mice, thus enhancing ketogenesis and the level of ketone bodies in plasma (33). Although the significance of this increased catabolism is still unclear, it might conceivably play a role in the peripheral fat wasting of lipoatrophy.

To determine whether this increased hepatic fat metabolism is a general property of all NRTIs or is restricted to some of them, we compared the mitochondrial and metabolic effects in mice of five major NRTIs and three of their combinations administered at therapeutic doses for 2 weeks. The present study provides evidence suggesting that increased hepatic fat metabolism is a unique feature of thymidine-based NRTIs and that these effects can be reproduced by β-aminoisobutyric acid (BAIBA), a thymine catabolite.

MATERIALS AND METHODS

Animals and treatments. Young (6- to 10-week-old) male Crl:CD-1(ICR) BR Swiss mice weighing 28 to 30 g each were purchased from Charles River (Saint-Aubin-lès-Elbeuf, France). Mice were either fed ad libitum a normal diet (A04 biscuits; UAR, Villemoisson-sur-Orge, France) or deprived of food for the last 48 h of treatment. All experiments were performed in agreement with the French national guidelines for the proper use of animals in biomedical research.

AZT and ddC were purchased from Sigma (Saint Quentin-Fallavier, France), while d4T and ddI (Videx) were kindly provided by Bristol-Myers Squibb (Rueil-Malmaison, France); 3TC (Epivir) was kindly provided by the pharmacy of Beaujon Hospital (Clichy, France). In the standard protocol, AZT (100 mg/kg of body weight/day), ddI (66 mg/kg/day), 3TC (50 mg/kg/day), d4T (13.5 mg/kg/

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TABLE 1. Concentrations of NRTIs in plasma[a]

Treatment (dose)	Concn in plasma (ng/ml)[b]				
	AZT	3TC	ddI	d4T	ddC
AZT (100 mg/kg/day)	1,408 ± 252 (10)				
3TC (50 mg/kg/day)		303 ± 39 (14)			
ddI (66 mg/kg/day)			63 ± 11 (10)		
d4T (13.5 mg/kg/day)				235 ± 31 (10)	
ddC (0.36 mg/kg/day)					<10 (10)
AZT-3TC	266 ± 71[c] (10)	376 ± 51 (10)			
d4T-3TC		413 ± 75 (10)		90 ± 21[c] (10)	
d4T-ddI			89 ± 31 (14)	79 ± 24[c] (16)	

[a] Mice were treated for 2 weeks with various NRTIs added to the drinking water. On the last day of treatment, blood was collected between 9 and 10 a.m., and concentrations in plasma were assessed as described in Materials and Methods.
[b] Results are means ± SEMs for the number of mice specified in parentheses.
[c] The value is significantly different from the value for NRTI given alone at a P of <0.05.

day), and ddC (0.36 mg/kg/day) were given in the drinking water for 2 weeks. These daily doses correspond to human dosages adjusted to body area, which is 0.026 and 0.30 m^2/kg of body weight in humans and mice, respectively. The quantity of NRTIs added to the drinking water was calculated on the basis of a daily liquid consumption of 5 ml per mouse. This daily liquid intake was unaffected by the treatments. For treatments combining two different NRTIs (AZT-3TC, d4T-3TC, or d4T-ddI), the drugs were mixed and given in the drinking water for 2 weeks. Mixing the different analogues in water gave stable solutions without precipitation. NRTI solutions were prepared every week and placed in drinking bottles. To assess NRTI stability, the drug concentrations in freshly prepared NRTI solutions (for ddI, ddC, AZT, and d4T-ddI) and after 1 week at room temperature were compared. NRTI concentrations determined after 1 week at room temperature ranged between 90 and 103% of the initial values.

In some experiments, BAIBA (a thymine catabolite) or higher doses of NRTIs were administered for 2 weeks in the drinking water.

NRTI concentrations in plasma. Plasma NRTI concentrations were determined by reversed-phase high-performance liquid chromatography as previously described (10), with minor modifications. Samples were extracted on C_{18} solid-phase extraction columns, and detection was done by measuring UV absorbance at 254 nm for d4T and ddI, at 260 nm for 3TC and ddC, and at 267 nm for AZT. Between-day and within-day variations of quality control samples of the different NRTIs were lower than 10%. The lower limit of quantification of the assay is 10 ng/ml for all analogues, and linearity is achieved at concentrations of 10 to 2,500 ng/ml for d4T, 3TC, and ddI and of 10 to 5,000 ng/ml for AZT.

Isolation of total DNA and slot blot hybridization. Total DNA was isolated from the liver, hind limb skeletal muscles, heart, brain, and epididymal white adipose tissue (WAT) by the phenol-chloroform method as previously described (30). To quantify mtDNA and nuclear DNA (nDNA), slot blot hybridization was performed as previously described (21, 39). Total DNA (200 to 400 ng) was blotted onto a Hybond-N^+ nylon membrane (Amersham, Les Ulis, France) and hybridized with an 8.6-kb mtDNA probe generated by long PCR and labeled by random priming (Multiprime DNA labeling system; Amersham). Membranes were stripped and hybridized with a mouse C_0t-1 nDNA probe (Gibco-BRL) as previously described (21, 39). mtDNA and nDNA were assessed by densitometric analysis of autoradiographs (21, 39).

In vivo formation of $^{14}CO_2$ from ^{14}C-fatty acids. The generation of $^{14}CO_2$ from [U-^{14}C]palmitate or [1-^{14}C]octanoate was assessed in fasted mice as previously described (29, 33). A tracer dose of [U-^{14}C]palmitate (3.7 μCi/kg; 4 nmol/kg) or [1-^{14}C]octanoate (4 μCi/kg; 69 nmol/kg) was given by gastric intubation in 0.2 ml of corn oil. Mice were placed individually in small plastic cages swept by an airflow of 50 ml per min. The outflow was bubbled into 60 ml of an ethanolamine–2-methoxyethanol mixture (30%:70%, vol/vol), an aliquot (3 ml) was removed at different times, and counts of $^{14}CO_2$ activity were determined. The exhalation of $^{14}CO_2$ was measured for 15 min after [1-^{14}C]octanoate administration and 120 min after [U-^{14}C]palmitate administration, as previously described (33).

β-Oxidation in isolated hepatic mitochondria. Liver mitochondria were prepared from fasted mice as previously described (28), and the β-oxidation of [U-^{14}C]palmitic acid by isolated mitochondria was assessed as previously described (29, 33). Briefly, mitochondria (containing 2 mg of protein) were preincubated at 30°C with 0.2 mM ATP, 50 μM L-carnitine, and 15 μM coenzyme A, with or without 2 mM KCN (which blocks mitochondrial β-oxidation). After 5 min, [U-^{14}C]palmitic acid (final concentration, 40 μM; 0.05 μCi/2 ml) was added with albumin, and the reaction was carried out for 10 min at 30°C. After the addition of 5% perchloric acid and centrifugation at 4,000 × g, ^{14}C-labeled acid-soluble β-oxidation products were counted in the supernatant. These products mainly represent ketone bodies and, to a small extent, citric acid cycle intermediates (28, 29).

Plasma ketone bodies, blood lactate and pyruvate, plasma triglycerides, cholesterol, and phospholipids. Plasma β-hydroxybutyrate and acetoacetate concentrations were measured as previously described (28). Blood lactate and pyruvate were assessed with commercial kits (Sigma diagnostics kits 826 and 726, respectively). The β-hydroxybutyrate/acetoacetate and lactate/pyruvate ratios were thus calculated for each animal. Plasma triglycerides, cholesterol, and phospholipids were measured with an automated analyzer (Hitachi model 717).

Northern blot analysis. Northern blot analysis was carried out as previously described (22). In this study, the cDNA of rat liver carnitine palmitoyltransferase I (CPT-I) was synthesized by reverse transcriptase PCR with primers 5′-TCCC CACTCAAGATGGCAGAGGCT-3′ (forward) and 5′-CTTCCGTGTGGCTC AGGGGTTTAC-3′ (backward) and was directly cloned into the pCRII vector (TA cloning kit; Invitrogen, Cergy Pontoise, France) according to the manufacturer's recommendations. Rat liver CPT-I probes hybridize specifically to mouse liver CPT-I mRNA (36). Total hepatic RNA was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction as described previously (16), and RNA integrity was assessed on an agarose gel before the Northern blot experiments. After hybridization with the CPT-I cDNA probe, nylon membranes (GeneScreen Plus; Perkin-Elmer Life Sciences, Wellesley, Mass.) were stripped and rehybridized with an 18S cDNA probe, which was used as a control probe. Autoradiographs were scanned, as previously described, in order to assess the relative amounts of the two RNA species (39). Finally, the CPT-I mRNA/18S rRNA ratio was calculated for each hepatic RNA sample.

Design of the experiments, presentation of the data, and statistical analysis. Due to the large number of animals, the effects of NRTIs (or their combinations) were usually studied in independent experiments, with one group of treated animals compared to one group of controls. Student's t test for independent data was used to assess the significance of the differences between control mice and treated mice in these experiments. To simplify the presentation of data in some figures and tables, control data are represented simply by the value 100 without the standard error of the mean (SEM), which was different in each individual experiment, while values for treated animals are expressed as percentages ± SEMs of the corresponding mean control values.

In some experiments, one control group was compared to several treatment groups. One-way analysis of variance followed by Dunnett's t test was employed for these multiple comparisons.

RESULTS

Plasma NRTI levels in mice. Plasma NRTI concentrations were assessed in mice treated for 2 weeks with AZT (100 mg/kg/day), 3TC (50 mg/kg/day), ddI (66 mg/kg/day), d4T (13.5 mg/kg/day), or ddC (0.36 mg/kg/day). As mice drink primarily during the night, blood was collected between 9 and 10 a.m. on the last day of the NRTI treatment (Table 1). Due to the low ddC doses administered, plasma ddC concentrations were be-

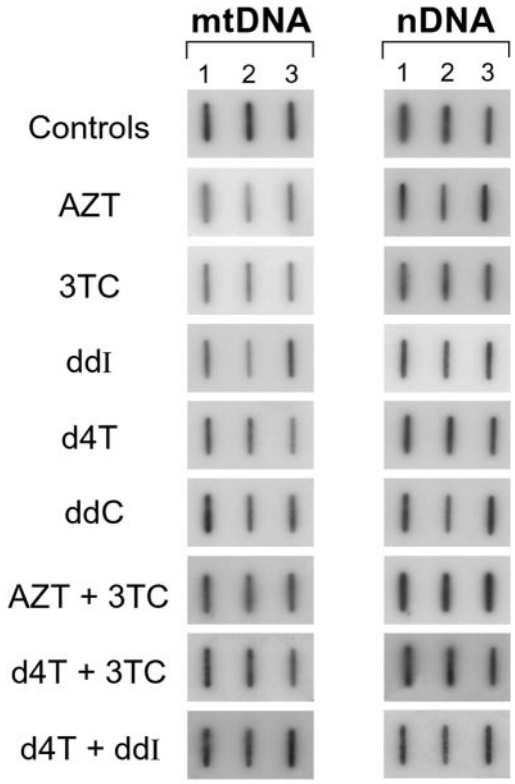

FIG. 1. Representative slot blots assessing hepatic mtDNA and nDNA levels. Groups of 10 to 16 mice were treated or not for 2 weeks with AZT (100 mg/kg/day), 3TC (50 mg/kg/day), ddI (66 mg/kg/day), d4T (13.5 mg/kg/day), ddC (0.36 mg/kg/day), or combinations of two NRTIs (same doses as for the single-drug treatments). Hepatic DNA (200 ng) was blotted on a nylon membrane, hybridized with an 8.6-kb mtDNA probe, stripped, and rehybridized with a mouse C_0t-1 nDNA probe. Representative slot blots for three mice are shown for each treatment. Quantitative data for all mice are shown in Table 2.

low the lower limit of quantification of the assay (10 ng/ml) for all mice. This result is similar to the plasma ddC concentrations for humans, which rarely exceed 10 ng/ml. Interestingly, AZT glucuronide was not found in the plasma of AZT-treated mice (data not shown), thus confirming that in mice, unlike humans, the glucuronidation of AZT is poor (1). Surprisingly, AZT concentrations were significantly lower in mice treated with the AZT-3TC combination than in mice treated with AZT alone (Table 1). Similarly, plasma d4T concentrations were lower in mice treated with the d4T-3TC or d4T-ddI combination than in mice treated with d4T alone (Table 1). These data suggest that absorption and/or pharmacokinetic interactions may have occurred, as discussed below.

Effects of NRTIs on mtDNA, blood lactate, and pyruvate. mtDNA and nDNA levels were determined by slot blot hybridization, and the mtDNA/nDNA hybridization ratio was used to assess mtDNA changes (Fig. 1; Table 2). None of the various treatments significantly decreased mtDNA in skeletal muscle, heart, brain, or WAT (Table 2). Hepatic mtDNA levels were decreased by 41, 32, and 30% with AZT, d4T, and 3TC, respectively (Table 2). In contrast, none of the three drug combinations had any effect on hepatic mtDNA (Table 2).

mtDNA depletion can decrease the activity of the mitochondrial respiratory chain to reduce the reoxidation of NADH into NAD^+. The increased NADH/NAD^+ ratio hampers the activity of mitochondrial pyruvate dehydrogenase, thus increasing blood pyruvate, which is, furthermore, increasingly interconverted into lactate due to the high NADH/NAD^+ ratio. We therefore assessed blood lactate and pyruvate in fed mice treated with the different NRTIs. We chose purposefully to measure the lactate and pyruvate levels in mice in the fed state because fasting can mask abnormalities of lactate metabolism induced by mitochondrial dysfunction (51). However, none of the eight different treatments with therapeutic doses of NRTI significantly increased blood lactate or blood pyruvate (data not shown). To determine if suprapharmacological doses of NRTIs could lead to disturbances in blood lactate or blood pyruvate, some mice were treated for 2 weeks with 500 mg of 3TC, d4T, or AZT/kg/day or 13 or 75 mg of ddC/kg/day. However, none of these high-dose treatments significantly increased blood lactate, blood pyruvate, or the lactate-to-pyruvate ratio. These results suggest that lactate and pyruvate metabolism is preserved in mice treated for 2 weeks with NRTIs, even when these drugs are given at very high dosages.

Effects of NRTIs on the in vivo formation of $^{14}CO_2$ from ^{14}C-fatty acids. Mitochondria play a primary role in fatty acid oxidation in diverse tissues, especially in subjects in the fasted state (32, 36). Therefore, we assessed the in vivo formation of

TABLE 2. mtDNA in different tissues after diverse NRTI treatments[a]

Treatment (dose)	mtDNA/nDNA hybridization ratio[b]				
	Liver	Muscle	Heart	Brain	WAT
None (controls)	100	100	100	100	100
AZT (100 mg/kg/day)	59 ± 8[c] (15/15)	138 ± 11 (14/8)	85 ± 7 (14/8)	113 ± 19 (14/8)	109 ± 11 (14/8)
3TC (50 mg/kg/day)	70 ± 8[c] (16/16)	85 ± 9 (14/8)	95 ± 5 (13/14)	75 ± 11 (13/10)	110 ± 15 (14/8)
ddI (66 mg/kg/day)	71 ± 10 (15/15)	96 ± 10 (14/8)	142 ± 16[c] (13/15)	91 ± 15 (15/13)	85 ± 14 (14/8)
d4T (13.5 mg/kg/day)	68 ± 7[c] (15/15)	93 ± 8 (13/12)	78 ± 6 (10/10)	93 ± 16 (15/10)	90 ± 16 (15/10)
ddC (0.36 mg/kg/day)	80 ± 13 (15/13)	77 ± 6 (9/7)	89 ± 5 (15/10)	72 ± 8 (15/10)	89 ± 15 (15/10)
AZT-3TC	83 ± 12 (12/14)	76 ± 10 (12/14)	75 ± 5 (12/14)	69 ± 12 (12/10)	118 ± 4[c] (12/10)
d4T-3TC	97 ± 5 (10/10)	134 ± 10 (8/8)	99 ± 5 (10/10)	112 ± 4 (10/10)	133 ± 8[c] (8/8)
d4T-ddI	99 ± 4 (12/12)	142 ± 11 (8/8)	89 ± 8 (13/13)	100 ± 7 (13/13)	86 ± 6 (8/8)

[a] Mice were treated or not with the different analogues for 2 weeks, and the mtDNA/nDNA hybridization ratios were determined as described in Materials and Methods.

[b] The mtDNA/nDNA hybridization ratio in treated mice (mean ± SEM) was expressed as the percentage of the mean mtDNA/nDNA hybridization ratio in the corresponding control mice. The numbers of mice in the control group/treated group are indicated within parentheses.

[c] The value is significantly different from the value for the corresponding control at a P of <0.05.

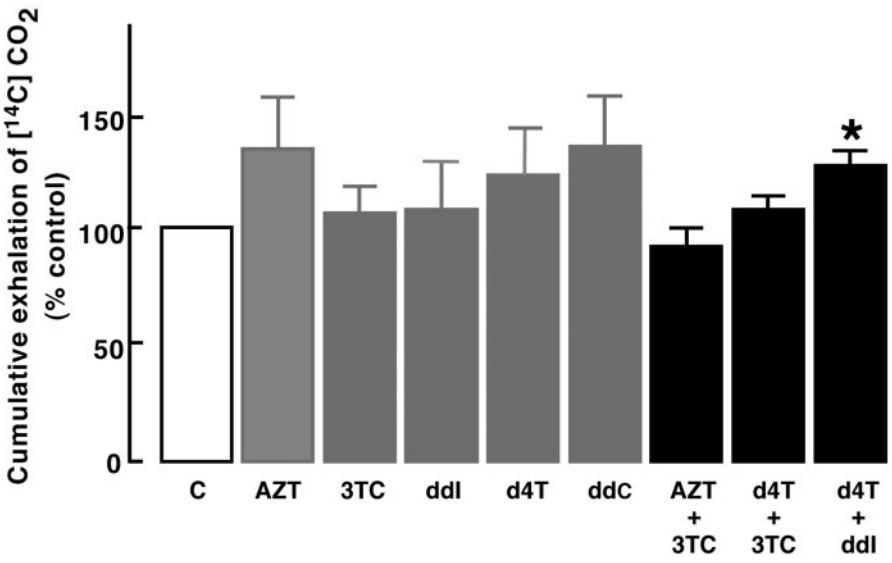

FIG. 2. Effect of NRTIs on the in vivo formation of $^{14}CO_2$ from [U-^{14}C]palmitate in mice. Mice were treated or not for 2 weeks with AZT (100 mg/kg/day), 3TC (50 mg/kg/day), ddI (66 mg/kg/day), d4T (13.5 mg/kg/day), ddC (0.36 mg/kg/day), or three combinations of two NRTIs (same doses as for the single-drug treatments) and fasted for the last 48 h of treatment. A tracer dose of [U-^{14}C]palmitate was administered, and $^{14}CO_2$ exhalation was measured for 120 min. Values for treated animals were expressed as percentages of the values for the corresponding controls. Each of the eight different control groups included 7 to 11 mice. Results for treated animals are means ± SEMs for 8 to 12 mice. The asterisk indicates a significant difference from results for the corresponding controls ($P < 0.05$).

$^{14}CO_2$ from [U-^{14}C]palmitic acid (a long-chain fatty acid) in mice treated with the different NRTIs for 2 weeks and fasted for the last 48 h of treatment (Fig. 2). Among the single-drug treatments, d4T, AZT, and ddC tended to increase the in vivo formation of $^{14}CO_2$ from [U-^{14}C]palmitic acid (by 23, 34 and 35%, respectively), but the differences from levels in control mice were not statistically significant. Among the drug combinations, only the d4T-ddI combination significantly increased the in vivo oxidation of [U-^{14}C]palmitic acid (by 27%).

We next assessed the in vivo formation of $^{14}CO_2$ from [1-^{14}C]octanoic acid (a medium-chain fatty acid) in mice treated for 2 weeks with d4T ($n = 9$ mice), AZT ($n = 10$), ddC ($n = 10$), or the d4T-ddI combination ($n = 10$) and fasted for the last 48 h of treatment. None of these treatments, however, had any effect on $^{14}CO_2$ exhalation (results not shown). Taken together, our results suggest that some NRTIs or their combinations may enhance long-chain fatty acid oxidation specifically.

Effects of NRTIs on plasma ketone bodies. In subjects in the fasted state, hepatic mitochondria oxidize long-chain fatty acids into ketone bodies (mainly β-hydroxybutyrate and acetoacetate), which are released into the circulation to serve as energetic fuels for the heart, skeletal muscles, kidneys, and brain (36). We therefore assessed the levels of ketone bodies in the plasma of mice treated for 2 weeks with therapeutic doses of NRTIs and fasted for the last 48 h of treatment (Fig. 3). Compared to controls, AZT (100 mg/kg/day) significantly increased total plasma ketone bodies by 46% ($P < 0.05$) due to a 55% increase in acetoacetate and a 44% increase in β-hydroxybutyrate (Fig. 3). Although d4T (13.5 mg/kg/day) also tended to slightly increase β-hydroxybutyrate (by 25%), the difference from levels in controls was not statistically significant. Other treatments had no effects, except for the d4T-3TC combination, which instead decreased plasma acetoacetate by 42% and plasma β-hydroxybutyrate by 38% (Fig. 3).

In a previous study, administration of 100 mg of d4T/kg/day for 6 weeks increased plasma acetoacetate by 73% and β-hydroxybutyrate by 107% in 48-h-fasted mice (33). In the present study, administration of 100 mg of d4T/kg/day for 2 weeks increased plasma acetoacetate by 12% (not significant) and plasma β-hydroxybutyrate by 69% ($P < 0.05$) in 48-h-fasted mice ($n = 14$). Thus, at doses between 13.5 and 100 mg/kg/day, d4T seems to increase plasma ketone bodies in a dose- and time-dependent manner. Similarly, the effects of AZT were dose dependent, since a fivefold-higher dose of AZT (500 mg/kg/day) for 2 weeks gave a larger increase in ketone bodies than the standard dose, with a 64% rise in acetoacetate ($P < 0.05$) and a 124% rise in β-hydroxybutyrate ($P < 0.01$) in 48-h-fasted mice ($n = 5$). We therefore examined whether higher doses of ddC or 3TC could unmask a latent ketogenetic effect of these NRTIs. However, increasing the dose of ddC from 0.36 mg/kg/day in the standard treatment to 13 or 75 mg/kg/day for 2 weeks failed to augment the levels of plasma ketone bodies (data not shown). Plasma ketone bodies were also unchanged in 10 mice treated with 500 mg of 3TC/kg/day. Altogether, these results indicate that AZT and d4T, but not ddC and 3TC, have a ketogenetic effect in mice.

Effects of BAIBA, a catabolite of thymine, on plasma ketone bodies. Because only AZT and d4T (two thymidine analogues) increased the levels of plasma ketone bodies, whereas ddI (an inosine analogue) or 3TC and ddC (two cytidine analogues) had no ketogenetic effects, we examined whether the ketogenetic effects of AZT and d4T could be reproduced by BAIBA, a β-amino acid generated during thymine catabolism (1, 19). Plasma ketone bodies were thus assessed in mice treated with BAIBA (10 or 100 mg/kg/day) for 2 weeks and fasted for the

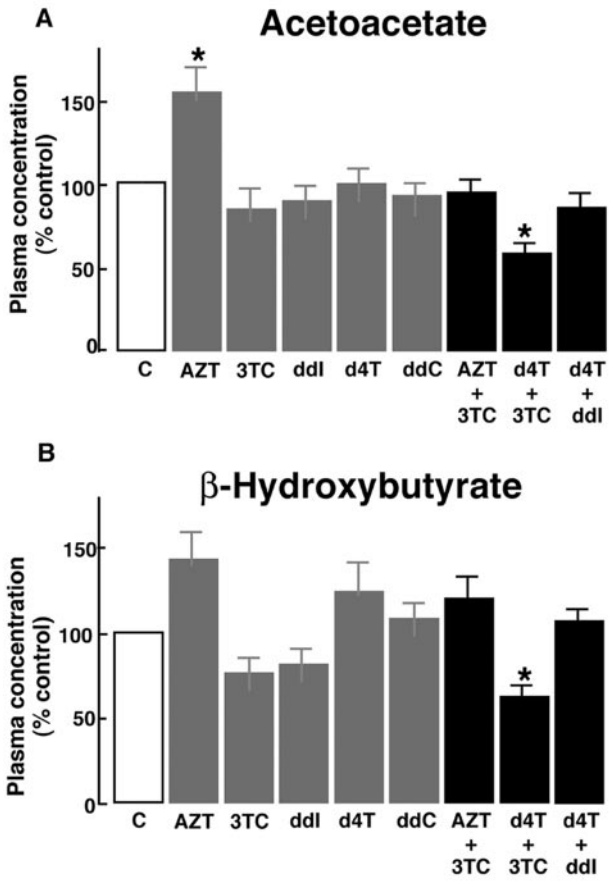

FIG. 3. Effect of NRTIs on plasma ketone bodies. Mice were treated or not for 2 weeks with AZT (100 mg/kg/day), 3TC (50 mg/kg/day), ddI (66 mg/kg/day), d4T (13.5 mg/kg/day), ddC (0.36 mg/kg/day), or different combinations of two NRTIs (same doses as for the single-drug treatments), and deprived of food for the last 48 h, before blood sampling for plasma acetoacetate and β-hydroxybutyrate measurements. Values for treated animals (grey and black bars) were expressed as the percentage of the values for the corresponding controls (white bar). Each of the eight different groups of controls included 6 to 13 mice. Results for treated animals are means ± SEMs for 7 to 15 mice. Asterisks indicate significant differences from the results for the corresponding controls ($P < 0.05$).

last 48 h of treatment (Fig. 4). Whereas the lowest dose had no significant effects, BAIBA at a dose of 100 mg/kg/day increased total ketone bodies by 64%, with a 64% increase in acetoacetate ($P < 0.05$) and a 64% increase in β-hydroxybutyrate (not statistically significant) (Fig. 4). Thus, these results suggested that BAIBA (or a downstream metabolite) may mediate, at least in part, the ketogenetic effects of d4T and AZT.

β-Oxidation of [U-^{14}C]palmitic acid in isolated hepatic mitochondria. Enhanced plasma ketone bodies can reflect enhanced hepatic ketogenesis due to an increased mitochondrial β-oxidation of fatty acids in the liver (33, 36). In a previous study, it has been shown that d4T at a dose of 100 mg/kg/day for 6 weeks increased [U-^{14}C]palmitic acid oxidation by 47% in isolated liver mitochondria (33), indicating that increased hepatic ketogenesis accounted, at least in part, for the high levels of plasma ketone bodies observed in d4T-treated mice (33). In the present study, [U-^{14}C]palmitic acid β-oxidation was assessed in liver mitochondria isolated from mice treated with AZT (100 mg/kg/day), d4T (13.5 mg/kg/day), and BAIBA (100 mg/kg/day) for 2 weeks and fasted for the last 48 h (Table 3). Our results showed that mitochondrial β-oxidation activity was increased by 16% with d4T, 31% with AZT ($P < 0.05$), and 88% with BAIBA ($P < 0.05$). Taken together, these results suggest that AZT and d4T increase hepatic mitochondrial β-oxidation and that this metabolic effect can be reproduced by BAIBA.

Expression of CPT-I in the liver. CPT-I, an enzyme located in the outer mitochondrial membrane, plays a key role in long-chain fatty acid oxidation and ketogenesis in the liver (22, 32, 40, 50). We therefore assessed hepatic levels of CPT-I mRNA in mice treated with AZT, d4T, or BAIBA at a dose of 100 mg/kg/day. In these experiments, we chose purposefully to study hepatic CPT-I expression in mice treated with 100 mg of d4T or BAIBA/kg/day because lower doses of these compounds induced only limited effects on plasma ketone bodies (Figs. 3 and 4) and hepatic mitochondrial β-oxidation (Table 3). Our results showed that AZT, d4T, and BAIBA signifi-

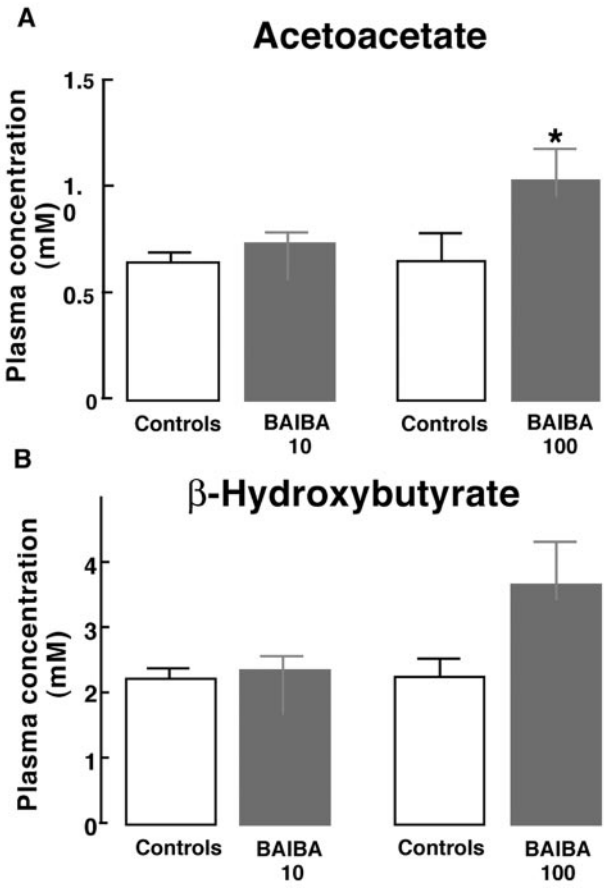

FIG. 4. Effect of BAIBA, a thymine catabolite, on plasma ketone bodies. Mice were treated or not for 2 weeks with BAIBA (10 or 100 mg/kg/day) in the drinking water and were deprived of food for the last 48 h of treatment before measurement of plasma acetoacetate and plasma β-hydroxybutyrate. Results (in millimolar concentrations) are means ± SEMs for 11 to 14 animals (for BAIBA at a dose of 10 mg/kg/day and the corresponding controls) or five to six animals (for the 100-mg/kg dose and the corresponding controls). The asterisk indicates a significant difference from the results for the corresponding controls ($P < 0.05$).

TABLE 3. Ex vivo effects of d4T, AZT, and BAIBA on [U-^{14}C]palmitic acid β-oxidation in liver mitochondria[a]

Treatment (dose)	Mice	Acid-soluble β-oxidation products (nmol/mg of protein/min)		
		Total activity	Mitochondrial activity	Peroxisomal activity
d4T (13.5 mg/kg/day)	Control	18.0 ± 4.1	16.0 ± 3.3	2.0 ± 0.5
	Treated	20.6 ± 4.4	18.5 ± 3.9	2.1 ± 1.4
AZT (100 mg/kg/day)	Control	15.4 ± 1.4	12.7 ± 1.2	2.7 ± 0.4
	Treated	19.3 ± 1.6	16.7 ± 1.6[b]	2.6 ± 0.5
BAIBA (100 mg/kg/day)	Control	20.3 ± 3.6	16.8 ± 2.7	3.5 ± 1.5
	Treated	33.8 ± 8.4[b]	31.6 ± 2.9[b]	1.9 ± 1.5

[a] Mice were treated or not with d4T, AZT, or BAIBA for 2 weeks and fasted for the last 48 h of treatment. Liver mitochondria were isolated and incubated at 30°C for 10 min with [U-^{14}C]palmitic acid, ATP, coenzyme A, and carnitine, with or without 2 mM KCN. The mitochondrial activity was determined as the difference between the β-oxidation activity measured without KCN (total activity) and that with KCN (peroxisomal activity). Results are means ± SEMs for eight mice.

[b] The value is significantly different from the value for the corresponding control at a P of <0.05.

cantly increased the CPT-I mRNA/18S rRNA hybridization ratio by 78, 51, and 53%, respectively (Fig. 5). In contrast, the CPT-I mRNA/18S rRNA hybridization ratio was unchanged in mice (four to six animals per group) treated for 2 weeks with ddI (66 mg/kg/day) or ddC (0.36 mg/kg/day). Altogether, our data suggest that the enhancement of CPT-I expression is specific for AZT and d4T and could be mediated, at least in part, by BAIBA.

Plasma triglycerides, phospholipids, and cholesterol. In a last series of investigations, we assessed the levels in plasma of triglycerides, phospholipids, and total cholesterol in mice treated with the different NRTIs. We found that ddC and the d4T-ddI combination significantly decreased plasma triglycerides by 32 and 50%, respectively (Table 4). In contrast, plasma triglycerides were significantly increased by 37% in mice treated with the AZT-3TC combination (Table 4). Plasma phospholipids and total cholesterol were not significantly affected regardless of the treatment used, although trends towards both lower phospholipids and lower total cholesterol (decreases ranging from 12 to 19%) were observed with 3TC, d4T-3TC, and d4T-ddI (data not shown).

DISCUSSION

To the best of our knowledge, this study is the first to compare the mitochondrial and metabolic effects of five major NRTIs and three of their combinations in an in vivo murine model. NRTIs were added to the drinking water in daily doses corresponding to human therapeutic doses per body area.

One conclusion of the present study is that, at least in mice, each NRTI combination should be considered a distinct treatment rather than the sum of two individual treatments. First, there were unexpected differences in plasma NRTI concentrations after single or dual treatments. Indeed, plasma AZT concentrations were lower when AZT and 3TC were coadministered, and plasma d4T concentrations were lower when d4T was administered with either 3TC or ddI than when either AZT or d4T was given alone (Table 1). However, the mixing of two drugs in the drinking water caused no precipitate, and the volumes ingested daily by the animals were identical for all treatments. Although more investigations are needed, these observations suggest that the intestinal absorption and/or pharmacokinetics of thymidine analogues (AZT and d4T) might be modified by the concomitant administration of some other NRTIs, at least in mice. Since such drug interactions have not been reported in humans, they might be mouse specific. Species differences are known with AZT, which undergoes glucuronidation in humans but not in mice (see Results and reference 1).

A second surprising observation from the present study is that, even when differences in drug concentrations were taken into account, the effects of treatments combining two NRTIs could not be predicted from the individual effects of each analogue (Fig. 1 to 3; Tables 2 and 4). For example, whereas 3TC alone, AZT alone, or d4T alone each significantly de-

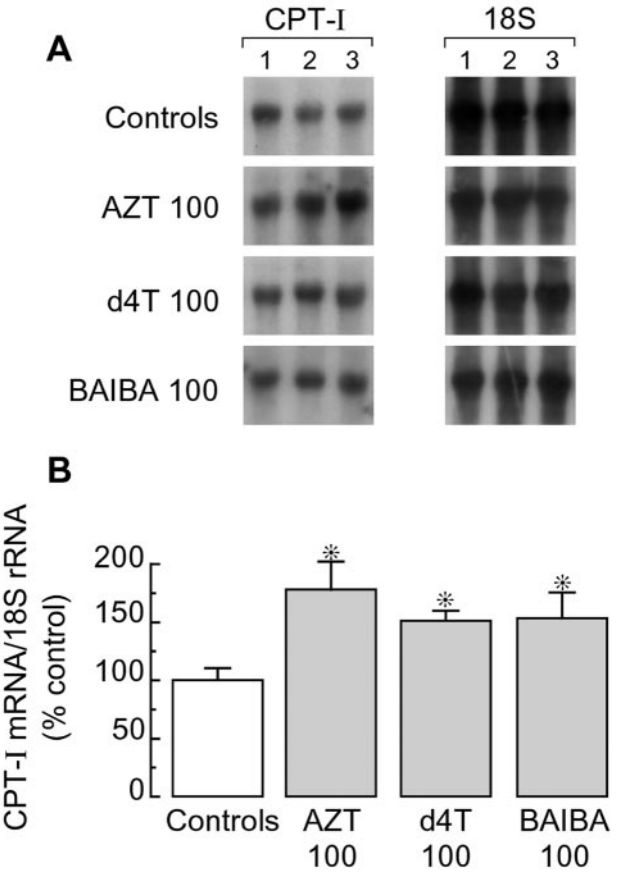

FIG. 5. Hepatic expression of CPT-I mRNA. (A) Representative Northern blots. Mice were treated or not with 100 mg of AZT, d4T, or BAIBA/kg/day in the drinking water for 2 weeks and were deprived of food for the last 48 h. Hepatic RNA was blotted on a nylon membrane, hybridized with a specific CPT-I probe, stripped, and rehybridized with an 18S cDNA probe. For each group of animals, representative blots for three different RNA samples (i.e., three mice) are shown. (B) CPT-I mRNA/18S rRNA hybridization ratios for the different groups of mice. Results (expressed as percentages of the control values) are means ± SEMs for 7 to 10 mice. Asterisks indicate significant differences from the results for the control mice ($P < 0.05$).

TABLE 4. Plasma triglycerides after diverse NRTI treatments[a]

Treatment (dose)	Plasma triglycerides (mmol/liter)[b]	
	Control mice	Treated mice
AZT (100 mg/kg/day)	2.2 ± 0.2 (12)	2.1 ± 0.2 (12)
3TC (50 mg/kg/day)	1.4 ± 0.2 (14)	1.1 ± 0.2 (14)
ddI (66 mg/kg/day)	1.9 ± 0.3 (10)	2.2 ± 0.2 (10)
d4T (13.5 mg/kg/day)	2.0 ± 0.2 (12)	1.8 ± 0.1 (14)
ddC (0.36 mg/kg/day)	1.9 ± 0.1 (10)	1.3 ± 0.2[c] (10)
AZT-3TC	1.6 ± 0.1 (15)	2.2 ± 0.2[c] (15)
d4T-3TC	2.1 ± 0.3 (8)	1.6 ± 0.2 (8)
d4T-ddI	1.6 ± 0.3 (10)	0.8 ± 0.2[c] (10)

[a] Mice were treated or not with the analogues for 2 weeks and fasted for the last 48 h of treatment.
[b] Results are means ± SEM for the number of mice specified in parentheses.
[c] The value is significantly different from the value for the corresponding control at a P of <0.05.

creased hepatic mtDNA, the administration of 3TC in combination with either AZT or d4T had no significant effects on hepatic mtDNA (Table 2). Although concentrations of d4T and AZT in plasma were lower after the dual treatments, 3TC concentrations in plasma were similar after all 3TC-containing treatments (Table 1) and would have been expected to also decrease hepatic mtDNA. Therefore, complex interactions seem to occur between different NRTIs. By the same token, Roche et al. (47) recently showed that the antiadipogenic effects of AZT in a murine preadipocyte cell line were eliminated when 3TC was added to AZT.

Another observation from the present study was that the administration of NRTI doses reproducing the human therapeutic doses on a body area basis had no effects on skeletal muscle, heart, brain, or WAT mtDNA and either no effects or only moderate effects on hepatic mtDNA after 2 weeks of treatment in mice (Table 2). Even the most active treatment (AZT) left 59% of residual mtDNA in the liver (Table 2). Since mtDNA encodes some of the polypeptides of complexes I, III, IV, and V of the respiratory chain, severe mtDNA depletion can decrease the activity of these complexes (5, 32). However, mtDNA must be severely depleted (to less than 20 to 30% of residual mtDNA) to impair mitochondrial respiration (24, 25). As expected, we found that complex I and complex IV activities were unchanged in the liver homogenates of mice treated for 2 weeks with ddI (66 mg/kg/day) or d4T (100 mg/kg/day) (results not shown), despite a ca. 30% decrease in hepatic mtDNA with both treatments (Table 2) (33). Unimpaired mitochondrial respiration likely explains why plasma lactate and plasma pyruvate were unchanged by the various NRTI treatments in the present study (see Results). Altogether, these results indicate that therapeutic doses of NRTIs do not impair mitochondrial function in mice after 2 weeks of treatment. Since NRTIs were administered for only 2 weeks, a greater effect on mtDNA after longer times of administration is not excluded. However, in mice treated with 500 mg of stavudine/kg daily for up to 6 weeks, hepatic mtDNA was decreased similarly after 1, 2, 4, or 6 weeks of treatment (33). With lower daily doses of stavudine (100 mg/kg), hepatic mtDNA decreased at 1 week but returned to normal levels at 6 weeks (33). Besides NRTIs, other factors may contribute to mitochondrial dysfunction in treated patients, including HIV infection (18, 34, 38, 45), cytokines, ethanol, and the administration of other drugs impairing mitochondrial function (9, 21, 27, 31, 32). Together with genetic predisposition (49), these added factors might play a role in individual susceptibility. Despite similar treatments, only a few treated patients develop severe mitochondrial dysfunction and related side effects (18).

The last important observation of the present study was that AZT and d4T (two thymidine analogues) increased plasma ketone bodies, whereas ddC and 3TC (two cytidine analogues) and ddI (an inosine analogue) had no significant effects on ketone bodies (Fig. 3) (see Results). Thymidine analogues can generate thymine, which is further degraded to BAIBA and other downstream catabolites (propionate, methylmalonate, and succinate). BAIBA is generated by rat hepatocytes incubated with d4T (19) and is found in the plasma of d4T-treated primates (19, 44). Circumstantial evidence also suggests the possibility of BAIBA generation from AZT by mouse tissues (1) or cultured hepatocytes from different species, including human hepatocytes (41). Interestingly, we found that BAIBA administration increased plasma ketone bodies in mice (Fig. 4), raising the possibility that the ketogenetic effects of AZT and d4T could be mediated, at least in part, by BAIBA (or a downstream metabolite). Since ketone bodies are exclusively synthesized in the liver after mitochondrial β-oxidation, we focused our investigations on this metabolic pathway. Increased fatty acid β-oxidation was found in hepatic mitochondria from mice treated with AZT, d4T, or BAIBA (Table 3), indicating that the increase in plasma ketone bodies could be due, at least in part, to increased hepatic ketogenesis. CPT-I is a key regulating enzyme involved in the mitochondrial β-oxidation of long-chain fatty acids and, thus, ketogenesis (23, 32, 40, 50). We found that hepatic levels of CPT-I mRNA were increased in mice treated with AZT, d4T, and BAIBA (Fig. 5). Thus, our data suggest that the effects of AZT and d4T on hepatic fatty acid oxidation and ketogenesis could be mediated, at least in part, by an increased expression of CPT-I. Interestingly, a 6-day treatment with d4T also increased CPT-I mRNA in cultured murine adipocytes (3T3-L1 cells) [O. P. Flint, R. Mulvey, S. Wang, W. Fenderson, W. P. Yang, and R. A. Parker, poster from the Third International Workshop on Adverse Drug Reactions and Lipodystrophy in HIV 2001, Antivir. Ther. **6**(Suppl. 4)**:**43-44, 2001]which express the liver CPT-I isoform (8). This finding suggests that the liver may not be the sole tissue where CPT-I, and thus fatty acid oxidation, can be up-regulated by thymidine analogues. To the best of our knowledge, it is not known whether deoxythymidine analogues also increase plasma ketone bodies in humans. However, in a study of a small number of HIV-infected children, there was a trend after a 10-h fast toward increased plasma ketone bodies in AZT-treated children compared to those in untreated or ddI-treated children (20).

Loss of peripheral fat tissue, sometimes associated with central adiposity, is a frequent complication of the new anti-HIV treatments combining protease inhibitors and NRTIs (6, 12, 14). Protease inhibitors induce insulin resistance and metabolic alterations in liver and adipose tissue, probably by interfering with sterol regulatory element-binding proteins (3, 46). In contrast, little is known of the mechanism(s) whereby NRTIs might contribute to peripheral fat wasting. Although NRTIs can deplete mtDNA in human adipocytes (42, 52), no correlation was found with lipodystrophy (15). An alternative hy-

pothesis is that increased fatty acid oxidation in the liver (as shown in the present study) and perhaps other organs [Flint et al., Antivir. Ther. **6**(Suppl. 4)**:**43-44] may cause a catabolic state contributing to adipose tissue wasting. We are currently performing investigations aimed at determining whether AZT, d4T, and BAIBA can affect fat homeostasis in lean and obese mice. Preliminary data suggest that in lean and obese mice these derivatives might change the amount of body fat and leptin expression in WAT. Whether these metabolic changes result from increased CPT-I expression and fatty acid oxidation, however, requires further investigation.

In summary, the present results suggest several conclusions. (i) The effects of NRTI associations cannot be predicted from the effects of each NRTI alone, as complex interactions seem to occur between diverse NRTIs in mice. (ii) NRTI treatments reproducing the human dose per body area have no effects, or limited effects, on mtDNA and do not modify blood lactate in mice after 2 weeks of treatment. (iii) Instead, AZT and d4T increase hepatic CPT-I mRNA expression, hepatic mitochondrial fatty acid β-oxidation, and plasma ketone bodies, and these effects are reproduced by BAIBA, a thymine metabolite. Whether these catabolic effects might play a role in lipoatrophy remains to be determined.

ACKNOWLEDGMENTS

Reine Note and Caroline Maisonneuve contributed equally to this work.

This work was supported in part by the University of Paris 7-Denis Diderot (Convention A007 relatif à l'appel d'offre SIDA) and Bristol-Myers Squibb. Reine Note was a recipient of a fellowship from Ensemble Contre le Sida.

We are indebted to Alain Truskolaski and Gérard Delrue for iconographical assistance and to Laurent Font for his help in animal care and treatments.

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